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13. ABSTRACT (Maximum 200 Words)

Oncogenic activation of ErbB2 and cSrc signaling pathways have been associated with worst prognosis and a more aggressive breast cancer phenotype. Here we explored the role of cSrc and ErbB2 as potentially contributing to estrogen receptor (ER) loss in ER protein negative (ER-) breast cancers. Our studies indicate that Src or proteasome inhibition increased ER levels in MCF-7, MDA-MB-361 and BT20. Furthermore, we have shown that Src activation cooperates with ligand to stimulate ER proteolysis and transcriptional activity. GW572016, an ErbB1 and ErbB2 inhibitor, decreased ER transcriptional activity in MCF-7 cells. ER- primary breast cancers and cell lines showed increased Src activity compared to ER+ cancers and cell lines, and the ER protein t1/2 was reduced in ER- breast cancer lines. Thus, these data provide a novel link between Src activation and the ER- tumor status and support a model whereby Src may promote transcription coupled ER proteolysis in breast cancer cells.

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INTRODUCTION

Newly diagnosed breast cancers are first assayed for estrogen receptor α (ERα) levels. Of these, 30% are found to fail to express detectable ER levels[1]. These breast cancers have worse prognosis, are resistant to antiestrogens and less responsive to chemotherapy [1;2]. Amplification of *ErbB2* and activation of receptor tyrosine kinase (RTK) signaling has been associated with poor patient prognosis [5] and has been correlated with ER negativity in primary breast cancers [3;4]. Both EGFR and ErbB2 can bind and synergize with cSrc to promote breast cancer cell proliferation, motility and survival [6]. cSrc in turn activates these RTK, thereby amplifying the signal. Since, I am studying how activation of RTK signaling is influencing ER activity and levels, I will briefly review the estrogen receptor pathway. Upon estrogen binding, the (ERα) recruits and activates Src leading to activation of Shc, MEK/MAPK, and PI3K/PKB [7]. Activation of these signaling kinases modulates cell cycle regulators to stimulate cell cycle progression [8;9] and these activated kinases phosphorylate ERα to positively influence ER dependent transcriptional activity [10;11]. Ligand stimulation also promotes ER degradation. The ER is rapidly degraded by ubiquitin-dependent proteolysis following estrogen binding [12]. This is a very tightly regulated process. However, this process has not been fully understood. In the present proposal we suggest that RTK and cSrc activation may synergize with endogenous estrogen to activate ligand dependent ER proteolysis in ER negative breast cancers. We also present evidence that oncogenic activation of RTK signaling is involved in tamoxifen resistance in ER positive breast cancer cells and abrogation of RTK signaling restores tamoxifen sensitivity by influencing cell cycle effectors.

BODY

Most of our previous findings and new findings are summarized in Appendix 1 (Manuscript that is being revised for submission to Journal of Clinical investigation). For the fist year I proposed to address task 1 and task 2, which involve further studying the role of cSrc on ER degradation by identifying a potential phosphorylation site. I have approached this task as will be described below and have been working on task 4 as this was requested by a reviewer when we sent our manuscript for publication.

Inhibition of Src increases ER protein levels in breast cancer lines with activated cSrc

The breast cancer line, BT-20 shows both cSrc and EGFR activation while ErbB2 and cSrc are activated in MDA-MB-361 [13]. BT-20 has been characterized as ER negative, and ER levels are reduced in MDA-MB-361. We confirmed increased Src kinase activity in MDA-MB-361 and BT-20 and decreased ER protein levels in these breast cancer cell lines (Figure 6B&E, appendix 1). As in MCF-7, ER accumulated in both BT-20 and MDA-MB-361 following serum and estrogen deprivation or proteasome inhibition (Figure 1). PP1 accumulated ER levels in MCF-7 but not in MDA-MB-361 or BT-20. This could be due to the toxicity of PP1 in these Src over expressing cell lines. Thus, we used a less toxic Src inhibitor, PD166326. When we used the Src inhibitor, PD166326, we observed abrogation of estrogen dependent ER degradation in MCF-7, MDA-MB-361 and BT-20 (Figure 1).

Critical role of an intact Y537 residue for ER activity and degradation.

The Tyrosine 537 residue in ER (Y537-ER) has been shown to be phosphorylated by cSrc in vitro[14]. We previously observed that mutation of Y537-ER to an alanine (Y537A-ER) was non-responsive

to estrogen (E) mediated ER degradation. To investigate if that was the case with the phenylalanine mutant (Y537F-ER), we constructed a Y537F-ER mutant. The F residue has a benzyl ring as is the non-phosphomimetic residue of the Tyrosine residue. Thus, we transfected wt-, Y537F- and Y537A- ER in MCF-7. We confirmed that ER-Y537A does not get degraded after estrogen stimulation (Figure 2). However, we observed that the ER-Y537F mutant was still degraded after estrogen addition (Figure 2). These results suggest that phosphorylation of Y537 alone might not be enough for ER proteolysis. It is possible that an intact tyrosine residue at that position is very critically structurally thus the difference in ER stability when that site is mutated to A or F. Furthermore, Katzellenbogen group has found that mutations of Tyr 537 to different residues affects ER transcriptional activity [15]. Taken together, these results offer strong evidence of the importance of that site in ER mediated transcriptional regulation and degradation.

Src binds to Y537A, Y537F and wt-ER with different affinities

Since the Y537 residue was very critical for degradation and transcriptional activity, We then proceeded to explore if Src interacted with ER and the role of Y537 if any in this interaction. By immunoprecipitation studies, we observed that Src bound more strongly in Y537A-ER mutant, followed by wt-ER and with the weakest and almost non-detectable interaction with Y537F-ER mutant (Figure 3). These results suggest that the region at Y537-ER is very important in mediating the interaction with Src. Therefore, it is possible that mutations at this site influence the structure of ER in such a way that it affects Src's affinity. Thus, Y537 seems to play role not only in degradation, transcription but also in Src's affinity.

Is Y537 phosphorylated

Src has been shown to phosphorylate Tyr 537 in vitro[14;16]. In order to try to observe in vitro phosphorylated ER, We used recombinant Src and recombinant ER and tried to phosphorylate ER in vitro, unfortunately we failed to detect phosphorylated ER under the conditions that we utilized. However, phosphorylation at this site has been controversial because of the difficulty to identify the actual phosphorylated site. Since Y537-ER is the only tyrosine site that has been postulated to be phosphorylated, we attempted to identify a phosphotyrosilated site in ER using a tyrosine specific antibody. However, we could not detect phosphotyrosilated ER when we immunoprecipitated ER from MCF-7. If Src phosphorylates ER, this could be due to the limitations of our experimental techniques or because this site is very unstable and thus, the difficulty in its detection. If ER is not phosphorylated by Src, then it is possible that Src may be mediating its effects in stability independent phosphorylation at this site.

Src and Estrogen cooperate to stimulate ER transcriptional activity

For many transcriptional factors, activation of transcriptional activity is linked to proteolysis of the transcription factor, thereby limiting extent of gene induction [17] Since our previous results demonstrated that Src activation resulted in estrogen stimulated ER proteolysis, we investigated effects of Src with and without estrogen on ER transcriptional activity. In proliferating cell, addition of estrogen or transfection of Src both increased ERE driven transcription within 4 hrs (fig 4A, appendix 1). Each of these treatments also caused a reduction in ER levels (fig 4B, appendix 1). Estrogen together with Src further increased ER transcriptional activity (fig4B, appendix 1). Thus ER cross talk with Src may contribute importantly not only to ER transcriptional activation, but also to ligand activated ER loss.

GW572016, an ErbB1 and ErbB2 inhibitor, inhibited ER transcriptional activity, increased p27 levels, decreased cyclin E/cdk2 activity resulting in cell cycle arrest.

Since we observed that Src transfection increased ER transcriptional activity, in order to determine if the opposite was also true, we used GW572016 which is an inhibitor of ErbB1 and ErbB2. Treatment with GW572016 alone reduced ER transcriptional activity by 38% in MCF-7 cells. Treatment of GW52016 in combination with Tamoxifen resulted in a cooperative reduction in ER activity in MCF-7 cells (Figure 4). Treatment for 48 hrs with GW572016 also resulted in increased p27 protein, decreased cyclin E/cdk2 activity and promotion of cell cycle arrest (data not shown). Thus inhibition of RTK signaling not only results in inhibition of ER transcriptional activity but also in cell cycle arrest in cooperation with tamoxifen in MCF-7 cells.

Figures

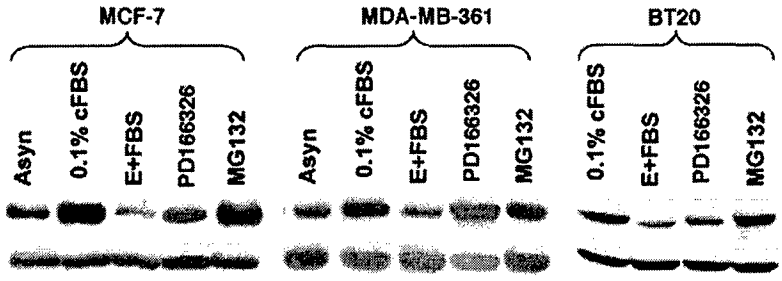

Figure 1. Cells were grown in 0.1% cFBS for 48 hrs prior to treatment with E plus 5%FBS in the presence or absence of the Src inhibitor, PD166326,or the proteasome inhibitor MG132 and ER levels were assayed by western blot

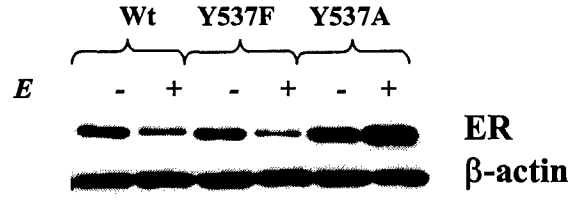

Figure 2. MCF-7 cells were transfected with wild type-, Y537F- Y537A-ER plasmids and then arrested for 48hrs by estradiol depletion. Estradiol was added for 6 hrs and cell lysates collected.

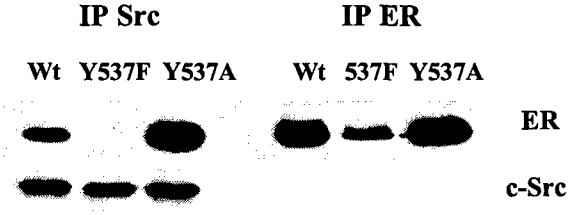

Figure 3. MCF-7 cells transfected with wt- , Y537F- or Y537A-ER were immunoprecipitated with a cSrc or ER antibody. ER and cSrc were assayed by Western blot.

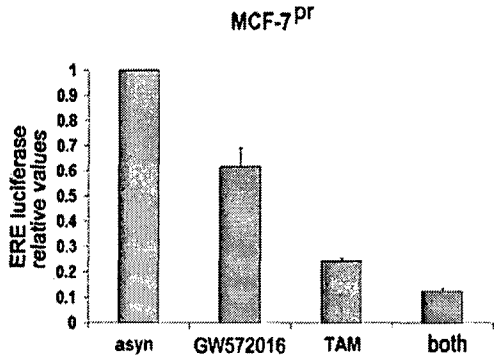

Figure 4. MCF-7 cells were transfected with 2xEREluc for 24 hours before treatment with DMSO control, 5 μmol/L lapatinib (GW572016), 1 nmol/L 4-OH-TAM or both drugs together for an additional 24 hours. Columns, mean of three independent experiments; bars, SE. Fold inhibition of ERE luciferase activity in untreated asynchronous control cells (asyn).

KEY RESEARCH ACCOMPLISHMENTS

Demonstrated that cSrc over expression cooperates with estrogen to stimulate ER degradation. Transfection of cSrc into MCF-7 resulted in a reduction of ER levels. Similarly over expression of ErbB2 reduced ER levels in MCF-7-Her2 clones.

Demonstrated that inhibition of cSrc using PP1 or PD166326 resulted in abrogation of Estrogen stimulated ER degradation in MCF-7, BT-20 and MDA-MB-361

Src transfection and estrogen cooperate to stimulate ERE transcriptional activity. These results give further evidence that activation of ER transcriptional activity is coupled to ER proteolysis.

There is increased Src activity in cell lines with reduced ER levels. The EGFR over expressing cell line, BT-20, and the ErbB2 over expressing cell line, MDA-MB-361 have greater Src activity compared to MCF-7 as assayed through a Src kinase assay and have reduced ER levels compared to MCF-7.

Demonstrated thatY537A-ER is a very stable protein compared to wt-ER and does not respond to estrogen dependent ER degradation. However, Y537F-ER was more sensitive to estrogen dependent ER degradation.

Demonstrated different binding affinities of ER to Src with mutations at Y537.

Demonstrated that inhibition of EGFR/ErbB2 upon treatment with GW572016 resulted in decreased ERE luciferase activity, increased p27, increased p27 bound to cyclin E/cdk2 and decreased cyclin E/cdk 2 kinase activity.

REPORTABLE OUTCOMES

Manuscripts

1. Article

   Src cooperates with estrogen to activate ligand dependent ERα proteolysis in human breast cancer. Isabel Chu, Angel Arnaout, Jun Sun, Arun Seth, Chris McMahon, Kathy Chun and Joyce Slingerland. Revising manuscript for submission to JCI, 2005

Poster presentations

1. cSrc and Her2 cooperate with estrogen to activate ligand dependent ERα proteolysis: implications for therapy of ER negative breast cancer. Isabel Chu, Jun Sun, Angel Arnaout and Joyce Slingerland. Keystone symposia, February-march, 2004

CONCLUSION

We have evidence that activation of RTK signaling is involved in ER transcriptional activation and ER degradation. Over expression of cSrc or ErbB2 results in decreased ER levels in MCF-7 cells. On the other hand, inhibition of Src using PP1 or PD166326 resulted in abrogation of estrogen dependent ER degradation. These findings implicate RTK signaling in ER degradation. We have also observed that inhibition of ErbB1/2 using the inhibitor GW572016 resulted in inhibition of ER transcriptional activity and cell cycle arrest. Thus RTK signaling has an important role in estrogen receptor signaling and cell cycle in mediating tumorigenesis in breast cancer cells. A better understanding of how RTK influence these pathways may shed new insights on the treatment of ER negative breast cancers or ER positive, tamoxifen resistant breast cancers.

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APPENDIX

Src cooperates with estrogen to activate ligand dependent ERα proteolysis in human breast cancer

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Nonstandard abbreviations used: Estrogen receptor (ER); ER positive (ER+); ER negative (ER-); Activation function (AF); Insulin (INS); charcoal stripped serum (cFBS); improved modified Eagle's medium (IMEM); Estrogen response element (ERE); cycloheximide (CHX); human pophobilinogen deaminase (h-PBGD); N-Acetyl-Leu-Leu-Norleucinal (LLnL)

ABSTRACT

Oncogenic cSrc activation may not only promote mitogenic signaling and aggressive proliferation, but also contribute to loss of detectable estrogen receptor α (ER) protein in a subset of ER negative (ER-) breast cancers. Real time PCR showed that all of 200 primary ER- and 50 ER+ breast cancers expressed ER mRNA with considerable variability and overlap in levels between groups. Investigation of mechanisms regulating ER levels indicates that Src cooperates with ligand to stimulate ER proteolysis: Src or proteasome inhibition increased ER levels and Src transfection stimulated both ligand activated ER transcriptional activity and ER proteolysis. ER- primary breast cancers and cell lines showed increased Src activity compared to ER+ cancers and cell lines, and the ER protein $t_{1/2}$ was reduced in ER- breast cancer lines. These data provide a novel link between Src activation and the ER- tumor status and support a model whereby Src may promote transcription coupled ER proteolysis in breast cancer cells.

Introduction

Estrogen regulates the proliferation and development of tissues expressing estrogen receptors and is implicated as a risk factor for the development of breast cancer. While there are two different types of ER, ERα and ERβ encoded by different genes (1-3), most data on ER expression in human breast cancers refers to ERα. This study investigates ERα exclusively and ER refers hereafter to ERα. ERα protein is assayed in newly diagnosed breast cancers because it is a clinically useful predictive and prognostic factor. About 33% of new breast cancers lack detectable ERα protein and have a worse prognosis than ER positive (ER+) breast cancers (4). ER negative (ER-) breast cancers are resistant to antiestrogens (5) and often develop resistance to chemotherapeutic agents (4). While estrogen is mitogenic for cultured ER+ breast cancer lines, ER- breast cancer lines proliferate in the absence of estrogen and ER- breast cancer has been thought to be estrogen independent.

Factors responsible for the ER- status of breast cancers remain largely unknown. *ER* gene alterations are too uncommon to account for the ER- phenotype (6;7). ER promoter hypermethylation has been implicated in a minority (up to 25%) of ER- breast carcinomas (6). Very few studies have assayed ER mRNA in primary breast cancers. Three early non-quantitative studies that assayed a total of 64 ER- tumors showed that 67-71% of ER- tumors express ER mRNA (8-10). These early observations raised the possibility that ER levels may be subject to important post-transcriptional or even post-translational control in human breast cancers.

The ER is a 66 kDa protein belonging to the nuclear hormone receptor superfamily of transcription factors (11). Upon ligand binding, ER dimerizes and associates with coactivators leading to transcriptional activation of estrogen response element (ERE) containing genes (12). ER contains two transcription activation functions (AF): AF-1 and AF-2. AF-1 can be phosphorylated and activated in a ligand independent manner following growth factor stimulation (13;14). AF-2 is activated by ligand stimulated changes in ER conformation (13;14). Transcriptional activation is influenced by the ERE sequence that affects ER-DNA binding affinity and by the phosphorylation state of ER that affects binding to coactivators.

In addition to transcriptional activation, ER-ligand binding rapidly activates cross talk with signaling cascades (for review see (15;16)). Estrogen-ER binding promotes a rapid and transient ER:cSrc interaction, cSrc activation (17;18), subsequent Shc phosphorylation and Ras-mitogen activated protein kinase (MAPK) signaling (17). In endothelial cells, liganded ER binds to the phosphoinositol 3' kinase (PI3K) leading to protein kinase B (PKB) activation (19). In other cell types, estrogen stimulates tripartite ER, cSrc and PI3K complex formation, and PKB and MAPK activation (20). Activation of signaling kinases following ligand-ER interaction can have mitogenic effects independent of transcription. It can also modulate ER phosphorylation to stimulate ER transcriptional activity (15;16). The ER can also be phosphorylated and activated in a ligand independent manner in response to signal transduction pathways resulting in ER-responsive gene activation. Several peptide growth factors including IGF-I (21), TGF-α (22) and EGF (23;24) activate signaling pathways that stimulate ER-dependent

transcription. Phosphorylation of amino terminal (19;23;25) and C-terminal (26-28) sites on the ER have been associated with increased ER transcriptional activity.

Estrogen-dependent ER activation rapidly stimulates ER-ubiquitination and ER-proteolysis (29-31). Unliganded ER is very stable, with $t_{1/2}$ of up to 5 days (29). Upon ligand binding, the ER $t_{1/2}$ drops dramatically to 3-5 h (29;31). The observations that ubiquitinated ER was found *in vivo* in the uterus in the presence of estrogen (29) and that proteasome inhibition abrogates estrogen dependent ER downregulation suggested a proteasomal pathway for ER degradation (30-32).

ER ubiquitination and proteasome activity are intimately linked to ER dependent transcriptional activation (32;33). Proteasome inhibitors and mutations that inhibit coactivator binding both abrogate ligand mediated ER proteolysis and ERE transcriptional activity (32). Ligand binding activates both ER dependent transcription and ER ubiquitination (33). Different ligands stimulate ER proteolysis to different degrees (34) and ubiquitin ligases MDM2 (35) and E6AP (36) can both stimulate estrogen induced transcriptional activity.

In the present study, ER mRNA levels were assayed in 250 primary human breast carcinomas using highly sensitive quantitative real-time PCR. All cancers expressed ER mRNA with significant overlap between ER mRNA levels in ER-positive and ER-negative breast cancers. Our investigation of mechanisms regulating ER protein levels suggest that crosstalk between ER and Src stimulates estrogen-dependent proteasomal degradation of the ER.

cSrc is a 60 kDa tyrosine kinase involved in regulation of proliferation, apoptosis, and metastasis (37). Increased levels or activity of cSrc have been observed in primary

breast cancers (38) but an association with ER status has not been reported. We observed that growth factors stimulate ligand mediated ER loss. The cooperation between growth factors and ligand to activate ER proteolysis appears to involve Src: cSrc overexpression led to a reduction in ER protein levels and $t_{1/2}$ and Src inhibition impaired ligand dependent ER proteolysis. Moreover Src and estrogen both stimulated ERE dependent transcription. ER negative breast cancer specimens and cell lines showed elevated cSrc activation compared to ER positive tumors and ER proteolysis was increased in ER-negative cell lines. Oncogenic Src activation may not only stimulate mitogenesis but also contribute to accelerated ER degradation in ER negative breast cancers.

Methods

Breast Cancer Specimens

Primary invasive human breast cancers snap frozen after surgical removal between 1994 and 1999 were obtained from the Sunnybrook and Women's College Hospital Cryopreserved Breast Cancer Tumor Bank. ER protein was quantitated by cytosolic assay at specimen accrual. This study was approved by the Sunnybrook Hospital Ethics Review Board

mRNA extraction and real time RT-PCR

Cellular mRNA was extracted from 100-150 gm of each of 300 carcinomas using TRIZOL as specified by the manufacturer (Molecular Research Center, Cincinatti, OH).

mRNA quality was visualized on ethidium gels. Only samples with OD 260/280 >1.3 and <2.1 were used for real time PCR quantitation. For 250/300 tumors, the extracted RNA was of sufficient quality to do quantitative analysis. 50 tumors were selected for strong ER protein expression (>30 fmol/mg protein), while 200 were ER protein negative (<5 fmol/mg protein). To verify equivalent quality of ER+ and ER- tumor derived mRNA, the expression of a house keeping gene, human porphobilinogen deaminase (h-PBGD), was quantitated in twelve ER-negative and ten ER positive RNA samples. These tumor RNA were subject to real time RT-PCR using the primer/hybridization probe mixture of the LightCycler h-PBGD Housekeeping Gene Kit (Roche). Serial dilutions of the h-PBGD RNA standared were used to generate a standard curve. Mean h-PBGD RNA expression values were compared in the 2 tumor groups using the Student's t test and did not differ significantly.

Primers for real time RT-PCR of ER mRNA were the exon 5 sequence 5'CTCCTAACTTGCTCTTGGACAG 3' and the exon 7 sequence 5'TCGGTTCCGC ATGATGAATC 3', respectively. PCR reactions were performed using the LightCycler System (Roche Molecular Biochemicals) and the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc.). The reactions contained 200ng RNA template, 1 μl each primer, 0.1U uracil DNA glycosylase (Epicentre), 10μl QuantiTect SYBR Green RT-PCR Master Mix (Qiagen Inc.), 0.2 μl QuantiTect RT Mix (Qiagen Inc.) and 3.5mmol $MgCl_2$. After incubation at 50°C for 20 minutes and initial activation at 95°C for 15 minutes, 45 cycles of 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 10 seconds were carried out. A program for the melting curve analysis was set at 95°C with a slope of 20°C per sec, 68°C with a slope of 20°C per second, and 95°C with a slope of 0.1°C.

Quantitation of ER mRNA From Real Time RT-PCR

A standard curve for ER mRNA quantitation was generated using serial dilutions of the full-length human ER cDNA plasmid, PCMV5hER-α (kindly provided by B. Katzenellenbogen). The crossing point values generated from plasmid PCR amplification were then plotted against the plasmid concentrations to generate a standard curve for quantitation of ER mRNA concentrations. Serial dilutions of MCF-7 ER mRNA amplified by real time PCR were then quantitated against the PCMV5hER-α plasmid standard curve. Alongside each tumor sample PCR amplification, a standard concentration of MCF-7 mRNA was used as an internal quantitative standard control for ER mRNA expression. Concentrations that could be evaluated from this standard curve ranged from 10 fg/μl to 1 μg/μl. All tumor samples had ER mRNA values in this range. The melting curve analysis obtained from each PCR reaction ensured that the fluorescence measured from each sample was attributable to ER amplification products rather than primer dimers. Water was used as a negative control in each PCR reaction while MCF-7 mRNA was the positive control. Differences in the amount of ER mRNA expressed in the ER-positive and ER-negative breast cancer samples were analyzed using the Student's *t*-test.

Sequencing of ER cDNA PCR Product

ER cDNA PCR products were visualized by 1.5% agarose gel electrophoresis for all tumor assays. For a subset of these, the PCR amplified ER cDNA was excised from gels and DNA extracted using the QIAquicke Gel Extraction Kit (Qiagen) and then

subjected to nucleotide sequencing. Reactions (20μl) contained 10 ng DNA, 3.2 pmol each of either the forward or reverse ER sequencing primer, Terminator Reaction Mix (ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit) and deionized water. The extension products were then precipitated with 3M sodium acetate and 95% non-denatured ethanol prior to sequencing with the ABI Prism 3100 Genetic Analyser.

Cell Culture

MCF-7 cells were grown in improved modified Eagle's medium (IMEM) (56). 5% FBS contains sufficient estrogenic hormone to support MCF-7 proliferation. Cells were estradiol depleted by transfer to IMEM-option Zn^{2+} phenol-red minus medium supplemented with 5% charcoal-stripped FBS (cFBS) for 48 h. Cells were depleted of both growth factors and estradiol by transfer to 0.1% cFBS fro 48 h. The ER negative BT-20 and the weakly ER positive MDA-MB-361 breast cancer lines were kindly provided by S. Parsons and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS and 1mM sodium pyruvate (39). The identity of these lines was verified and confirmed by karyotype analysis. To assay effects of growth factors on ER levels, estrogen with or without 5% FBS or 5% cFBS alone was added to MCF-7 cells that had been starved of estrogen and growth factors in 0.1% cFBS for 48 h.

Plasmids and Transfection

An activated human cSrc vector, PCI-Src Y530F, was kindly provided by D. Fujita (University of Calgary). Asynchronous MCF-7 cells were transfected with either PCI-Src Y530F or empty PCI vector (10 μg) using lipofectamine PLUS™ (GIBCO). A vector encoding activated human Her2, CMVHer2-Glu, was kindly provided by R. Kerbel (University of Toronto). MCF-7 cells were transfected with Her2-Glu and stable clones selected.

Flow cytometric analysis

Cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 2 hours and then fixed, stained with anti-BrdU-conjugated FITC (Becton Dickison) and propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software as described in (56).

Antibodies

The anti-ER monoclonal antibody, H222, was kindly supplied by G. Greene (University of Chicago); polyclonal ER antibody HC-20 was obtained from Santa Cruz; and monoclonal Src antibody, GD11, from Upstate Biotechnology. Antibodies against MAPK, phosphosphorylated, activated MAPK (P-MAPK), PKB and phosphorylated activated PKB (PKB-P) were obtained from New England Biolabs and to β-actin from Sigma.

Immunoblotting and cycloheximide chase

Cells were lysed in ice cold D/RB buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 2.5 mM EGTA pH 8.0, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween-20, 1 mM PMSF, 0.1 mM Na_2VO_4, 0.5 mM DTT, and 0.02 mg/ml each of aprotinin, leupepsin and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis. 20-100 μg protein per lane was used for Westerns. The ER $t_{1/2}$ was determined by cycloheximide (CHX) chase experiments with addition of 100 μg of CHX at t=0. Lysates were collected for immunoblotting at the indicated time points thereafter. To quantitate ER protein on Western blots, densitometry of 3 experiments was performed using the Molecular Dynamics Imaging system and Image Quant software and results graphed.

Effects of MEK and PI3K inhibition on ER stability

To assay effects of MEK or PI3K inhibition on ER levels increasing concentrations of UO126 (Promega) (0.1-10 μM) or LY294002 (Promega) (0.5-8 μM) were added to asynchronous MCF-7 cultures for 48 h prior to recovery for immunoblotting or flow cytometry. In addition, estradiol and growth factor depleted MCF-7 cultures were treated with either 10μM UO126 or 8 μM LY294002 for 30 min prior to stimulation with 17β-estradiol for 6 h, followed by recovery for immunoblotting or flow cytometric analysis.

Src kinase assay

Human breast cancer lines or pulverized primary human breast tissue samples were lysed in ice cold NP40 lysis buffer (1 % NP40, 150mM NaCl, 20mM Tris/HCl, 1 mM NaF, 1 mM PMSF, 0.1 mM Na_2VO_4, 1 mM EDTA pH 8.0, and 0.02 mg/ml each of

aprotinin, and leupepsin). Lysates were clarified by centrifugation and 200 μg of protein lysate was used for immunoprecipitation with 1 μg Src antibody, GD11 (Upstate Biotechnology). For cSrc kinase assays, inactivated enolase and $^{32}P\gamma$-ATP were incubated with the immunoprecipitated cSrc as previously described (57). Reactions were resolved by SDS-PAGE, and radioactivity incorporated in the enolase substrate was quantitated using a Molecular Dynamics PhosphoImager and ImageQuant software.

ERE luciferase assays

MCF-7 cells were grown in a 24 well plate and transfected with 500 ng a plasmid bearing 2 tandem ERE (2 x ERE luc), 50 ng phRL-TK luc and 100 ng cSRc-Y530F using lipofectamine/plus (Gibco) as specified by the manufacturer. Cells were treated with 10 μM PP1 and/or E for 4 h before lysate collection. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and Beckman Coulter LD 400.

Results

ER negative breast cancers express ER mRNA

ER mRNA expression in tumor samples was quantitated by comparing real time RT-PCR crossing point values to those of a standard curve generated from serial dilutions of an ER cDNA plasmid (Figure 1a). All of 50 ER+ and 200 ER- tumors showed detectable ER mRNA (Figure 1b shows representative samples). Quantitation of the housekeeping gene human porphobilinogen deaminase (h-PBGD) expression showed

that the quality of extracted mRNA did not differ between the two groups. The scatter plot of ER mRNA values in the two groups of tumors is shown in Figure 1c. The distribution of different ER mRNA concentrations in ER+ and ER– cancers are graphed in Figure 1d. The mean ER mRNA concentration in ER+ cancers was 1.14×10^3 (range 1.02×10^{-1} to 1.19×10^4) fmol/μg RNA. That in ER- cancers was 1.27×10^3 (range of 4.55×10^{-2} to 3.56×10^4) fmol/μg. While the lowest and highest ER mRNA concentrations were similar and the mean ER mRNA values did not differ significantly between the two breast cancer groups ($p>0.50$), the modal ER mRNA value in the ER- tumors was approximately one log lower than in the ER+ cancers (Figure 1d).

Serum growth factors synergize with estrogen to activate ER proteolysis

As a baseline for further investigation, we established that estradiol addition to estrogen-deprived MCF-7 cells stimulated a rapid reduction of ER protein that was impaired by proteasome inhibition with N-Acetyl-Leu-Leu-Norleucinal (LLnL) (Figure 2a). The ER protein $t_{1/2}$ was >24 h in estrogen depleted MCF-7. Within 6 h after addition of estrogen, the ER $t_{1/2}$ fell to 5 h (Figure 2b). A significant reduction in ER $t_{1/2}$ was also noted within 1 h of ligand addition (not shown).

Receptor tyrosine kinases can cross talk with liganded ER to phosphorylate the ER and activate its transcriptional activity (21). To test if signaling cross talk could modulate ligand dependent ER proteolysis, we tested if serum or insulin could cooperate with ligand to stimulate ER loss. MCF-7 cells were deprived of both growth factors and estrogen by transfer to 0.1% charcoal stripped FBS (cFBS) for 48 h. As in Figure 2a, estrogen caused a reduction in ER within 6 h. Addition of 5% FBS together with

estrogen reduced ER levels more rapidly (Figure 2c). Growth factor stimulation by transfer to 5% cFBS without added estrogen was not sufficient to trigger ER proteolysis (lane 4, Figure 2c). Addition of 10 μg/ml insulin (INS) together with estrogen to cFBS starved cells also stimulated a more rapid loss of ER than did estrogen alone (Figure 2d). Insulin alone had no effect on ER levels in the absence of estrogen. Thus, growth factors present in serum may activate signaling pathways that cooperate with estradiol to activate ER proteolysis.

cSrc cooperates with estrogen to stimulate ER degradation

Liganded ER is known to recruit and activate cSrc (17). Treatment of MCF-7 with the Src inhibitor, PP1, caused a dose dependent accumulation of ER over 48 h (Figure 3a). cSrc inhibition by PP1 also partly impaired the fall in ER levels observed when estrogen and growth factor starved cells were exposed to serum together with estradiol (Figure 3b). Thus, cSrc activation may contribute to ligand dependent ER proteolysis. Transfection of activated *cSrc* (PCI-Src Y530F) caused a reduction in ER levels within 48 h (Figure 3c). Despite a transfection efficiency of only 60%, at 24 h after *cSrc* transfection, the ER $t_{1/2}$ was reduced to 9 h compared to 14 h in asynchronously proliferating MCF-7 transfected with empty vector (Figure 3d).

Her2 overexpression is frequent in human breast cancers and interaction between Her2 and cSrc leads to synergistic activation of both kinases (39;40). Transient transfection of MCF-7 with activated Her2 (CMVHer2-Glu) increased cSrc kinase activity within 24 h (Figure 3e). In stable MCF-7 clones expressing CMVHer2-Glu ER levels were reduced (Figure 3f). Transfection of activated cSrc into a Her2

overexpressing MCF-7 line, H5b, further reduced ER levels (Figure 3g). Thus Her2 and cSrc may cooperate to activate ER proteolysis.

Src and estradiol cooperate to stimulate ERE transcriptional activity

For many transcription factors, activation of transcriptional activity is linked to factor proteolysis, thereby limiting the extent of gene induction (41). Since Src transfection stimulated ER proteolysis, we assayed effects of Src with and without estradiol on ER transcriptional activity. In proliferating cells, addition of 10^{-8}M estradiol or transfection of Src both reproducibly increased ERE driven transcription within 4 hours (Figure 4a). Each of these treatments also caused a modest reduction in ER levels (Figure 4b). Estrogen together with Src transfection further increased ER transcriptional activity. ER consistently fell by 2.8 fold as assayed by densitometry 4 h after Src transfection and estradiol stimulation (Figure 4b). When ERE luciferase activity relative to available ER was measured ("correcting" for differences in ER levels at 4 h), the effects of Src activation and estrogen stimulation on ERE based transcriptional activation appear to be greater than additive (Figure 4c). Thus, ER cross talk with Src may contribute importantly not only to ER transcriptional activation, but also to ligand activated ER loss.

MAPK and PKB do not stimulate ligand mediated ER proteolysis

Treatment of asynchronous MCF-7 with the MEK inhibitor, U0126, for 48 h caused a dose dependent reduction in ER levels (Figure 5a). Moreover, in serum and estrogen deprived MCF-7 cells, addition of estrogen together with U0126 led to a greater loss of ER (Figure 5b) and a shorter ER $t_{1/2}$ (not shown) than that seen following estrogen

alone. Thus, activation of MAPK or of other MEK effectors may oppose ligand stimulated ER proteolysis. It is noteworthy that MAPK activation also opposes ligand mediated retinoid X receptor proteolysis (42).

Treatment of asynchronous MCF-7 with the PI3K inhibitor, LY294002, did not affect ER levels (Figure 5c). In serum and estrogen deprived MCF-7, transfer to 5% cFBS plus 10^{-8}M 17β-estradiol stimulated PKB activation and G_0 to S phase progression. LY294004 inhibited PKB phosphorylation and cell cycle progression, but did not affect estrogen-mediated ER loss (Figure 5d & e). Thus, estrogen stimulated ER proteolysis does not require PI3K/PKB activation or cell cycle entry. These results also suggest that ligand dependent ER proteolysis is not cell cycle dependent.

Reduced ER protein levels and stability in breast cancer lines with activated cSrc

The breast cancer line, BT-20, shows both cSrc and EGFR activation while Her2 and cSrc are activated in MDA-MB-361 (39). BT-20 has been characterized as ER negative, and ER levels are reduced in MDA-MB-361 (39). The identities of MDA-MB-361 and BT-20 lines were confirmed by karyotyping. ER mRNA was detected in MCF-7, BT-20 and MDA-MB-361 by non-quantitative RT-PCR (Figure 6a). ER protein levels and $t_{1/2}$ were reduced in both BT-20 and MDA-MB-361 compared to MCF-7 (Figure 6b-d) and cSrc kinase activity was increased (Figure 6e). The ER $t_{1/2}$ was 14 h in asynchronous MCF-7, 9 h in MDA-MB-361 and 5 h in BT-20. As in MCF-7, ER accumulated in both BT-20 and MDA-MB-361 following serum and estrogen deprivation or proteasome inhibition (Figure 6f). Thus, estrogen stimulated ER loss is active in these lines and ER levels are regulated by proteolysis not only in ER positive MCF-7 but

also both of these (ER negative and weakly ER positive) lines. Note that more protein was loaded and immunoblots were exposed longer for the MDA-MB-361 and BT-20 compared to MCF-7 in Figure 6f.

cSrc is activated in ER negative primary breast cancers

Although cSrc activation has been reported in a limited number of human breast cancers, it has not been correlated with ER levels (38). cSrc kinase activity was assayed in 18 ER- and 22 ER+ primary human breast cancers. The tumor ER status determined at diagnosis by cytosolic ligand binding assay was verified by ER immunoblotting. β-actin blotting verified equal loading and equal protein input into the cSrc kinase assays (Figure 7). While cSrc levels did not differ significantly among ER+ and ER- tumors, elevated cSrc activity was observed in 78% (14/18) of ER- breast cancers. Only 18% (4/22) of ER+ tumors showed Src activity above controls (Figure 7).

Discussion

ER gene deletions, rearrangements and point mutations are rare and cannot explain the lack of ER protein in up to one third of newly diagnosed breast cancers(6;7). ER mRNA has not been extensively quantitated in primary breast cancers. Relatively insensitive dot blot, Northern and standard RT-PCR analyses demonstrated ER mRNA in a majority (60-70%) of ER- tumors(8-10). Using more sensitive real time RT-PCR, Iwao et al detected ER mRNA in all of 52 ER- primary breast cancers (43). The present more comprehensive real time PCR quantitation showed all of 200 ER- breast cancers

expressed ER mRNA. ER mRNA concentrations varied considerably within ER+ and ER- cancers and showed significant overlap between the two tumor types. While the mean ER mRNA concentrations did not differ significantly, the modal distribution of ER mRNA concentrations was lower in ER negative cancers.

Although microarray studies have shown ER- breast cancer correlates with reduced ER gene expression (44-46), these studies do not demonstrate that ER- cancers lack ER mRNA. Array studies compared individual breast cancer ER mRNA to a reference of mixed cRNAs pooled from ER+ and ER- tumors (44) or to the average signal from all tumors (45;46) and show that on average, ER- tumors have lower ER gene expression than that in reference controls. These findings are thus not inconsistent with our data. Other array studies show variable ERα gene expression in both ER+ and ER- tumors (47). The discordance between ER mRNA and protein status in ER– cancers and overlap of values and the large range of ER mRNA concentrations we observed in both ER+ and ER- cancers point to important ER regulation at post-transcriptional levels.

The present investigation suggests that cross talk between liganded ER and Src may not only regulate ER transcriptional activity, but also activate ER proteolysis. 20-30% of primary breast cancers show HER2/erbB-2 amplification(48) or increased expression of the EGFR(49) and both are strongly associated with an ER- status(48;49). In breast cancer cells, cSrc can bind phosphorylated Her2 or EGFR promoting synergistic activation of these signaling pathways that stimulate breast cancer cell proliferation, motility and survival (39).

In addition to modulating breast cancer cell proliferation and survival, our data suggest that cSrc activation following estrogen-ER binding contributes to ligand

dependent ER proteolysis. cSrc inhibition impaired ligand dependent ER loss. Src transfection accelerated ER proteolysis and reduced ER levels and constitutive Her2 and cSrc together further reduced ER levels. Moreover, a majority of primary ER negative tumors showed elevated cSrc activity. The ER-, EGFR overexpressing BT-20 and weakly ER+ Her2 amplified MDA-MB361 breast cancer lines both show constitutive cSrc activation. ER protein was detectable but unstable in these lines and increased following growth factor and estrogen deprivation. Thus ER levels in these lines are affected by estrogen. These data raise the intriguing possibility that receptor tyrosine kinase and cSrc activation may underly the accelerated ER proteolysis observed in BT-20 and MDA-MB-361. The specific effect of Src inhibition on estrogen and serum mediated ER loss could not be evaluated in the these lines because treatment of steroid and growth factor starved cells with PP1 together with serum and estrogen caused apoptosis.

Although a majority of primary ER- breast tumors (78%) showed cSrc activation, it is noteworthy that cSrc was not consistently elevated in all. This may be an artifact of cryopreservation or the ER- phenotype may be Src independent in some cancers. That four ER+ cancers also showed high cSrc activity suggests that other factors in addition to cSrc activation may be required for ER loss *in vivo*.

Signaling pathways that activate many transcription factors, including c-Jun, c-Myc and E2F-1 also trigger their ubiquitin dependent degradation (41). This provides an efficient mechanism to limit transactivator availability and function. Ubiquitylation of some factors is required for their transcriptional activity (41;50). Transcription factor

ubiquitylation may influence coactivator/repressor binding (41) and coactivators enhance ubiquitylation of certain transcription factors (51).

Ligand mediated proteolysis accounts for the turnover of most nuclear receptors including progesterone receptors (52), thyroid hormone receptors(53), retinoic X receptors (54) and ERα (29-31). Proteasome inhibition decreases estrogen-ER-transcriptional activity despite an increase in ER abundance (32). Components of the ubiquitin pathway including the ubiquitin ligases E6AP (36) and MDM2 (35) and the 19S proteasomal subunit, Rpt6/SUG1 exhibit ER co-activator activity (55). ER mutations that impair co-activator binding abrogate ligand-stimulated ER degradation. Thus, co-activator binding may regulate not only transcriptional activity but also ligand mediated ER degradation(32). Reid et al recently demonstrated that ER cycles on and off of an ERE promoter together with ubiquitin ligases and Rpt6/SUG1 (33). Ligand binding increased ER-ERE binding and recruitment of RNA polymerase II and modified the pattern of ubiquitin ligase binding. Ubiquitinated ER dissociated from DNA and accumulated in nuclear matrix after proteasome inhibition.

While proteolytic degradation of the ER after ERE firing may allow re-loading of the promoter, it may potentially serve a more global role in regulating the abundance and overall activity of the ER. Ligand stimulated ER ubiquitination may regulate transcriptional activity through transcription coupled ER proteolysis. Moreover, constitutive ER activation could potentially lead to reduced ER levels, due to constitutive ER proteolysis.

We postulate that ligand mediated ER cross talk with cSrc or cSrc dependent kinases may lead to phosphorylation events that facilitate ER binding to

coactivators/components of the proteolytic machinery and ER dependent transcriptional activation coupled to ER proteolysis. cSrc transfection increased both the transcriptional activity and ER protein loss induced by estrogen. How specific Src dependent ER phosphorylation events modulate the profile of coactivator binding, ERE selection and ER proteolysis will require further investigation.

ER phosphorylation mediated by cross talk with different signaling pathways could theoretically lead to recruitment of different coactivators or ubiquitin pathway components, thereby changing not only the spectrum of ER dependent genes expressed but also the efficiency of ligand stimulated ER proteolysis. This could influence patterns of ER regulated gene expression in different tissues and during progression of breast and other estrogen sensitive cancers. During breast cancer progression, Src activation may alter coactivator binding, shifting ER transcriptional targets to profiles that promote oncogenic change.

ER- cell lines have been thought to be estrogen insensitive since they do not require estrogen for growth. This and the clinical observation that ER- breast cancers resist antiestrogenic therapies (5) have led to the belief that ER- tumors are estrogen insensitive. Our data raise the concern that ER- breast cancers may indeed be stimulated by estrogen *in vivo*. Constitutive ER proteolysis in ER- cancers may not reflect extinguished ER-dependent transcription, but rather a shift to constitutive activation of different ER transcriptional targets. Our preliminary interrogation of gene expression profiling data from ER+ and ER- breast cancers (47) reveals ER dependent gene expression in both tumor types (J. Sun and J. Slingerland, unpublished). The therapeutic implications of this work are potentially very significant. Our data provide a

new rationale for the development of Src inhibitors in the molecular therapeutics of ER- breast cancer. Inhibition of RTK and cSrc signaling in ER- breast cancers may alter the profile of ER-co-activator binding, inhibit ER proteolysis and restore responsiveness to antiestrogenic therapies. The development of pre-clinical models to test this hypothesis is warranted.

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Figure Legends

Figure 1

ER- and ER+ breast tumors express ER mRNA by real time RT-PCR. (**a**) Top: agarose gel electrophoresis of different concentrations of ER plasmids. Bottom: a standard curve of plasmid concentrations and "crossing points" in the ER RT-PCR experiment. (**b**) Agarose gel electrophoresis of ER cDNA and h-PBGD of 5 ER + and 5 ER- tumors. C represents no mRNA, water control. (**c**) Scatter-plot diagram of ER mRNA concentrations in ER+ and ER- breast cancers. Mean is represented with a horizontal line. (**d**) Histogram plots the comparison of the frequency of ER mRNA concentration levels in ER+ and ER- tumors. Each ER mRNA value was rounded to the nearest logarithm value.

Figure 2

Estradiol stimulates proteasomal ER degradation. (**a**) asynchronous MCF-7 were arrested by 17β-estradiol depletion. Immunoblot of ER 6 h after addition of the proteasome inhibitor, LLnL, prior to 17β-estradiol addition to estrogen-depleted MCF-7. For all blots, β-actin confirmed equal loading. (**b**) The $t_{1/2}$ of ER protein was assayed in both 17β-estradiol depleted cells and at 6 h after addition of estrogen. Cycloheximide (CHX) was added at t=0, and cell lysates were collected for ER immunoblotting at the indicated times. Densitometric analysis of 3 CHX chase experiments was performed and ER amounts graphed vs time to determine the ER $t_{1/2}$. Linear regression of

cycloheximide chase shows ER $t_{1/2}$. (**c**) cells were estrogen and growth factor starved for 1 day by culture in medium with 0.1% cFBS and then treated with 17β-estradiol alone, 5% cFBS +E or 5% cFBS alone. Lysates were collected and immunoblotted for ER 6 hours later. (**d**) MCF-7 were grown in 0.1% cFBS and stimulated E alone or Insulin (INS) and E for 6 h and then ER assayed by immunoblotting.

Figure 3

cSrc activation stimulates ER degradation. (**a**) the Src kinase inhibitor, PP1, was added to proliferating MCF-7 and ER levels assayed 48 h later. (**b**) quiescent, serum and estrogen deprived MCF-7 were transferred to medium containing 5% FBS plus E, with or without added PP1 and ER levels assayed by immunoblotting. (**c**) MCF-7 were transfected with constitutively active cSrc-Y530F or with empty vector control and assayed for ER and cSrc by immunoblotting. (**d**) MCF-7 were transfected with cSrc-Y530F for 24 h, CHX was added and cell lysates collected at intervals and immunoblotted for ER. Densitometric analysis of 3 CHX chase experiments was performed. ER loss with time was graphed and linear regression performed to determine the ER $t_{1/2}$. (**e**) MCF-7 were transfected with an activated Her2. Her2 was immunoblotted and cSrc kinase assayed 24 h later. (**f**) MCF-7 clones stably transfected with an activated Her2 expressed lower ER levels than vector alone controls. (**g**) The stable Her2 clone, H5b, was transiently transfected with cSrc for 24 h and ER immunoblotted.

Figure 4

cSrc synergizes with estrogen to activate ER transcriptional activity. MCF-7 were transfected with a luciferase construct containing an estrogen-responsive promoter (2xERE) and with a cSrc-Y530F plasmid or empty vector control. Cells were treated with 10 nM 17-β estradiol (E) for 4 hours and samples were harvested after 4 hours of treatment. (**a**) Shows ERE luciferase activity 4 hours post-transfection. (**b**) Shows the levels of ER and Src before (C) and 4 hours after Src transfection, estradiol treatment or both. (**c**) Relative ERE luciferase is graphed taking into account the reduced levels of ER available at 4 hours post transfection.

Figure 5

MAPK and PI3K do not stimulate estrogen dependent ER degradation. (**a**) asynchronously growing MCF-7 cells were treated with 0.1, 1 or 10 μM of the MEK inhibitor, UO126 for 48 hr and then ER and activated phosphorylated MAPK (MAPK-P) levels were assayed. (**b**) quiescent, serum and estrogen-deprived MCF-7 cells were treated with estrogen and 5% FBS with or without 10 μM UO126 for 6 hr and ER levels assayed by immunoblotting. (**c**) MCF-7 cells treated with increasing concentrations of the PI3K inhibitor, LY294002, for 48 hr were collected and assayed for ER and activated PKB (P-PKB). (**d** & **e**), estrogen and serum deprived MCF-7 cells were stimulated with estrogen plus 5% FBS or estrogen plus 5%FBS together with 8 μM LY294002 for 6 and 18 hr followed by ER and PKB immunoblotting. At 18 hr, cell cycle distribution was assayed by flow cytometry.

Figure 6

Breast cancer lines overexpressing cSrc have reduced ER. (**a**) ER mRNA was assayed in MCF-7, MDA-MB361 and BT20 by RT-PCR. Reaction products were resolved and visualized by Gel Doc. (**b**) The ER protein was assayed in asynchronous MCF-7, MDA-MB-361 and BT20 by immunoprecipitation of ER from 1 mg cell lysate followed by immunoblotting. (**c**) The ER $t_{1/2}$ was assayed in asynchronous MCF-7, MDA-MB-361 and BT20 by CHX chase with CHX addition at t=0. Cell lysates were collected for ER immunoblotting at the indicated times. β-actin blotting confirms equal loading. (**d**) Densitometric analysis of 3 CHX chase experiments and linear regression was performed to determine the ER $t_{1/2}$. (**e**) cSrc kinase activity was assayed in asynchronous MCF-7, MDA-MB-361 and BT20. (**f**) Estrogen (E) activates ER proteolysis in MCF-7, MDA-MB-361 and BT-20. After growth in 0.1% cFBS for 48 h MCF-7, MDA-MB-361 and BT-20 were stimulated with E plus 5%FBS in the presence or absence of the proteasome inhibitor MG132 and ER levels assayed by western blotting 6 h later.

Figure 7

ER negative primary breast carcinomas show cSrc activation. Snap frozen primary tumors were pulverized, lysed and clarified by centrifugation. cSrc kinase activity was assayed in 22 ER positive and 18 ER negative tumors as described in Methods. The ER status of these tumors was verified by ER immunoblotting and β-actin verified equal loading.

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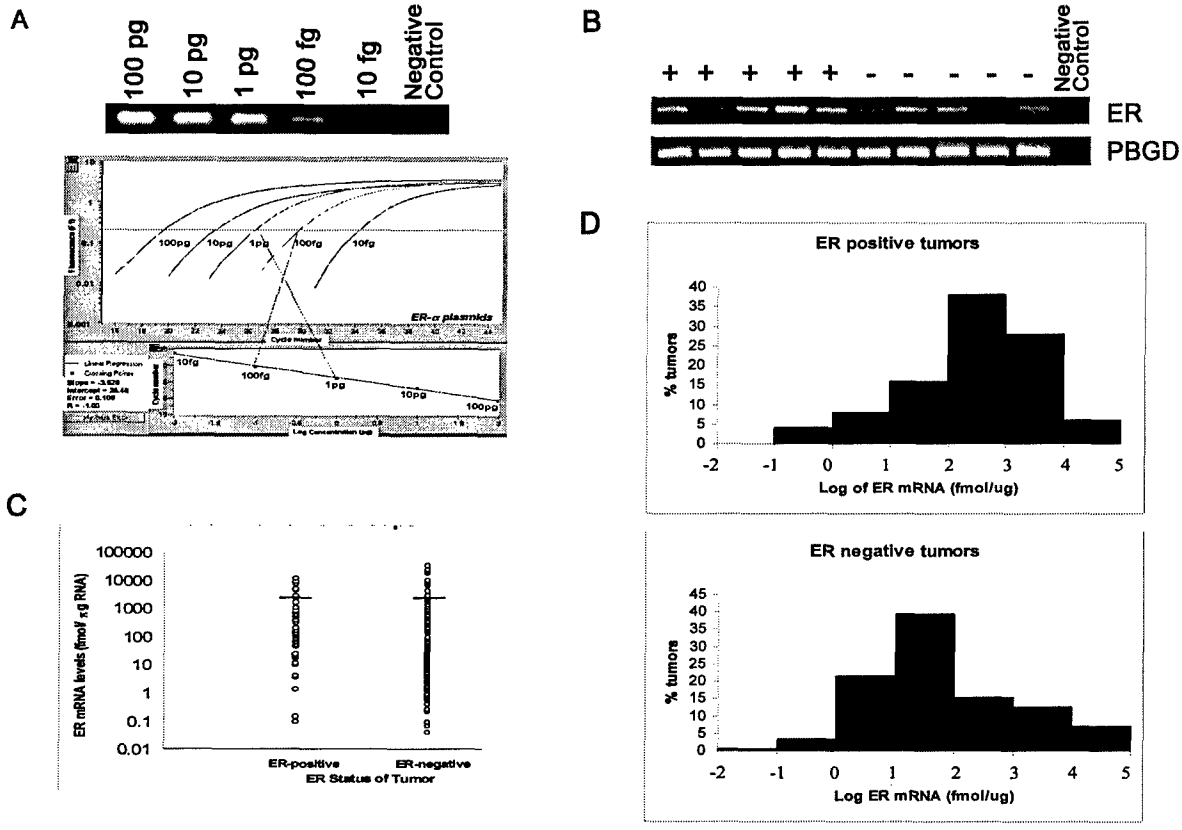

A
100 pg
10 pg
1 pg
100 fg
10 fg
Negative Control
B
+ + + + + - - - - -
Negative Control
ER
PBGD
C
ER mRNA levels (fmol/ µg RNA)
100000
10000
1000
100
10
1
0.1
0.01
ER-positive
ER-negative
ER Status of Tumor
D
ER positive tumors
% tumors
Log of ER mRNA (fmol/ug)
ER negative tumors
% tumors
Log ER mRNA (fmol/ug)

Figure 1

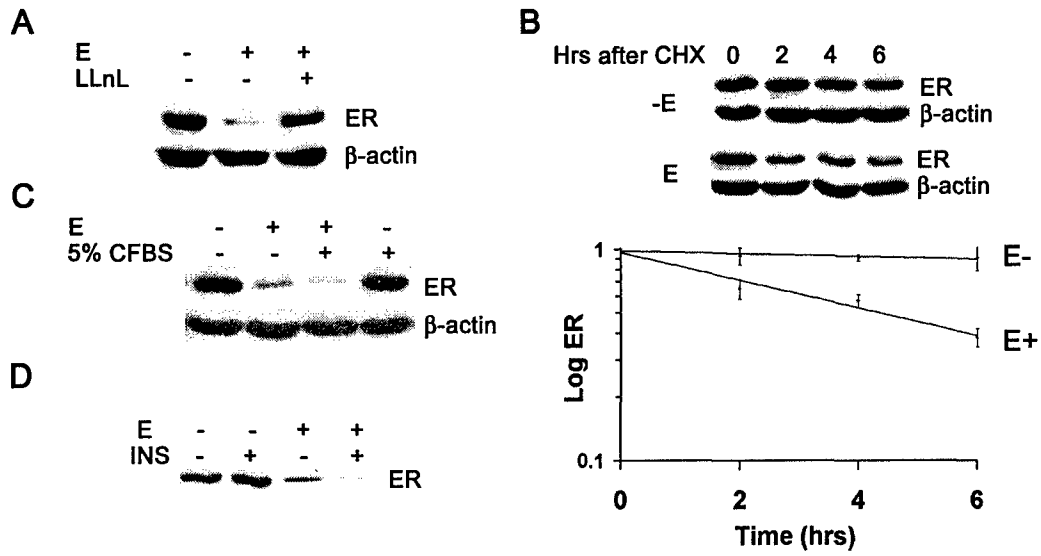
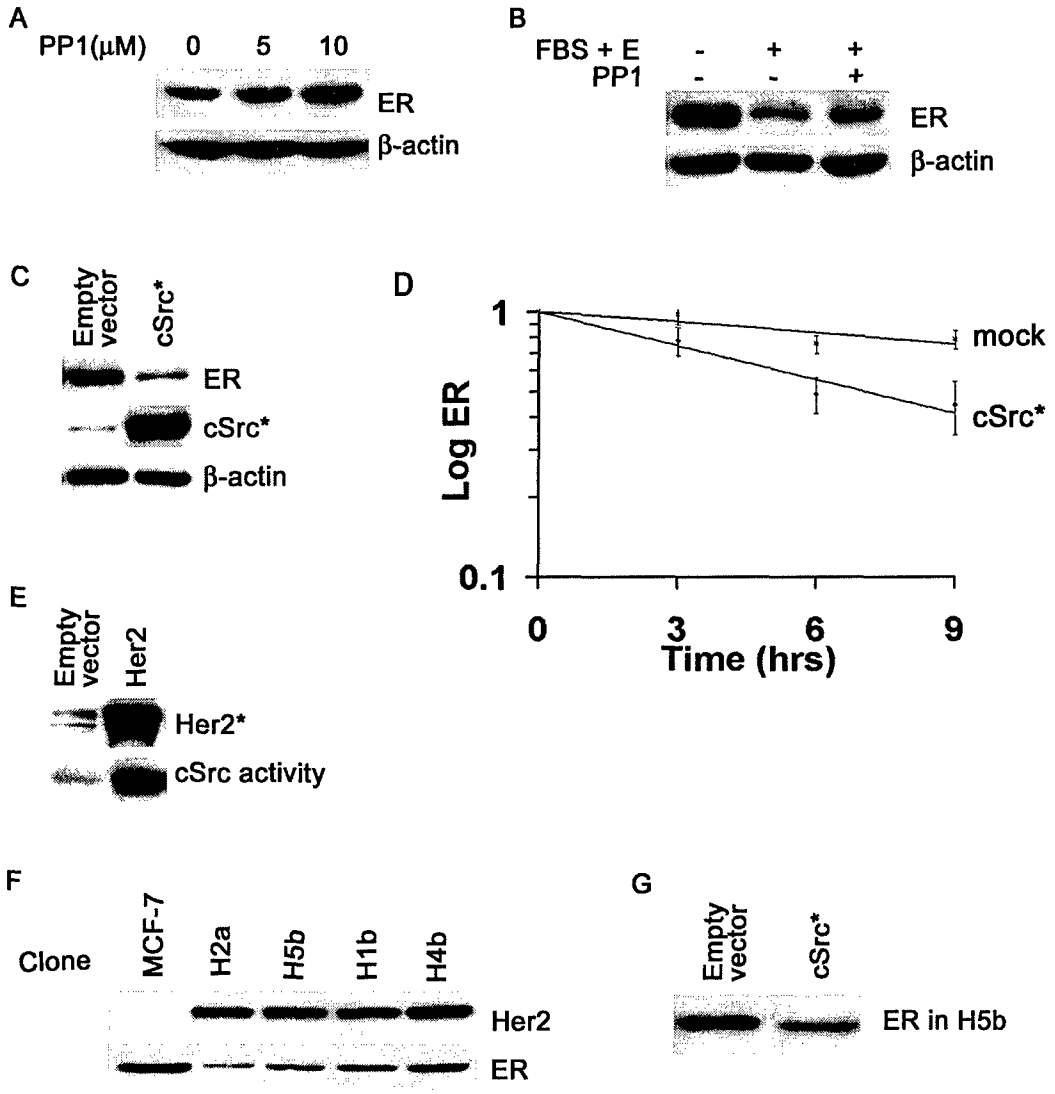

A
E - + +
LLnL - - +
ER
β-actin
B
Hrs after CHX 0 2 4 6
-E
ER
β-actin
E
ER
β-actin
C
E - + + -
5% CFBS - - + +
ER
β-actin
D
E - - + +
INS - + - +
ER
Log ER
1
0.1
0 2 4 6
Time (hrs)
E-
E+

Figure 2


A
PP1(μM) 0 5 10
ER
β-actin
B
FBS + E - + +
PP1 - - +
ER
β-actin
C
Empty vector
cSrc*
ER
cSrc*
β-actin
D
1
0.1
Log ER
mock
cSrc*
0 3 6 9
Time (hrs)
E
Empty vector
Her2
Her2*
cSrc activity
F
Clone
MCF-7
H2a
H5b
H1b
H4b
Her2
ER
G
Empty vector
cSrc*
ER in H5b

Fiigure 3

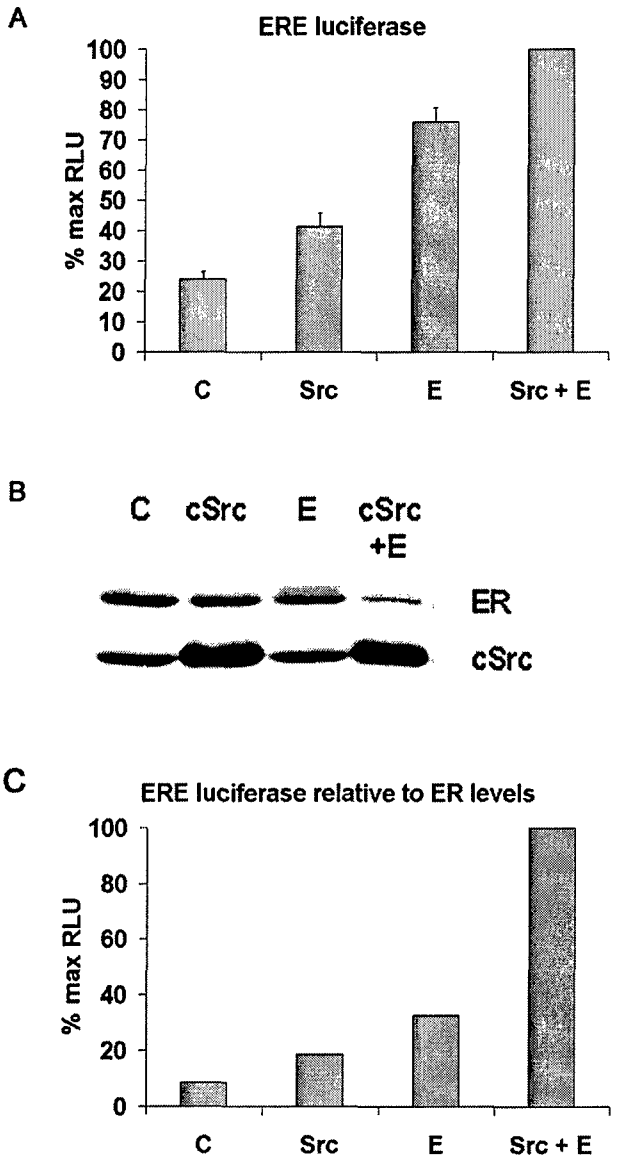

A
ERE luciferase
% max RLU
100
90
80
70
60
50
40
30
20
10
0
C
Src
E
Src + E
B
C
cSrc
E
cSrc
+E
ER
cSrc
C
ERE luciferase relative to ER levels
% max RLU
100
80
60
40
20
0
C
Src
E
Src + E

Figure 4

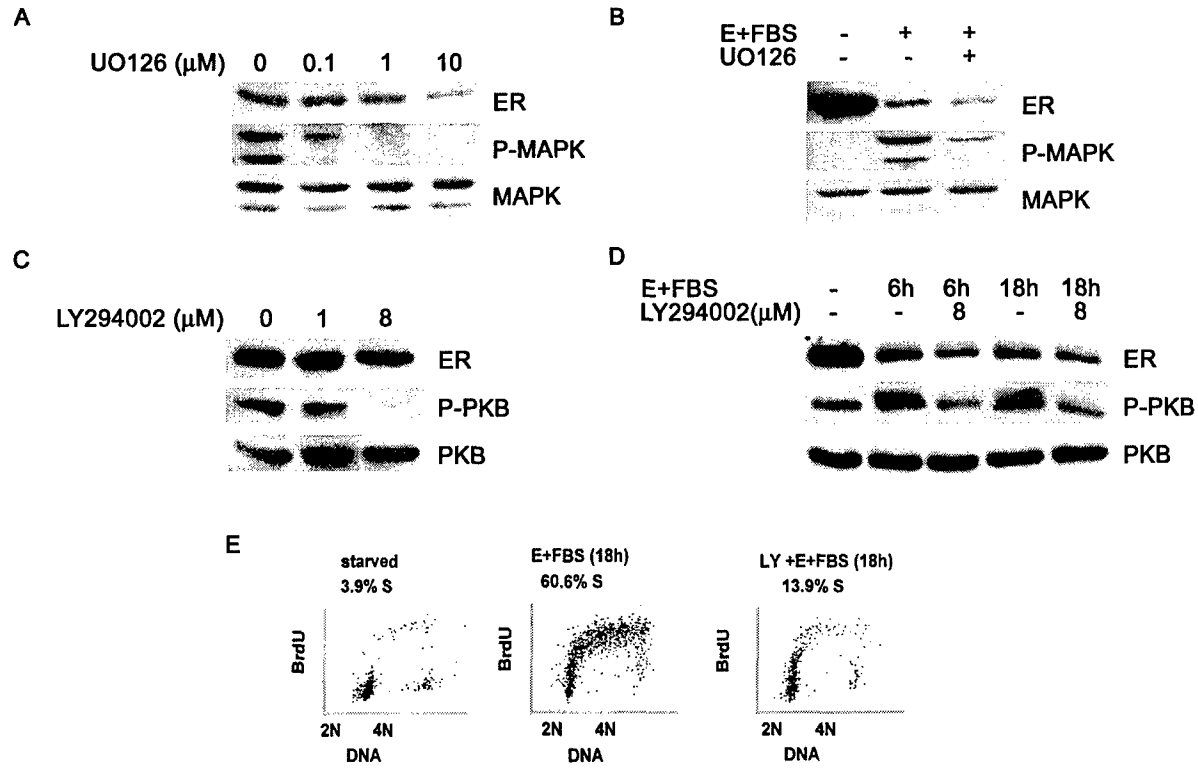

A
UO126 (μM) 0 0.1 1 10
ER
P-MAPK
MAPK
B
E+FBS - + +
UO126 - - +
ER
P-MAPK
MAPK
C
LY294002 (μM) 0 1 8
ER
P-PKB
PKB
D
E+FBS - 6h 6h 18h 18h
LY294002(μM) - - 8 - 8
ER
P-PKB
PKB
E
starved
3.9% S
E+FBS (18h)
60.6% S
LY +E+FBS (18h)
13.9% S
BrdU
2N 4N
DNA

Figure 5

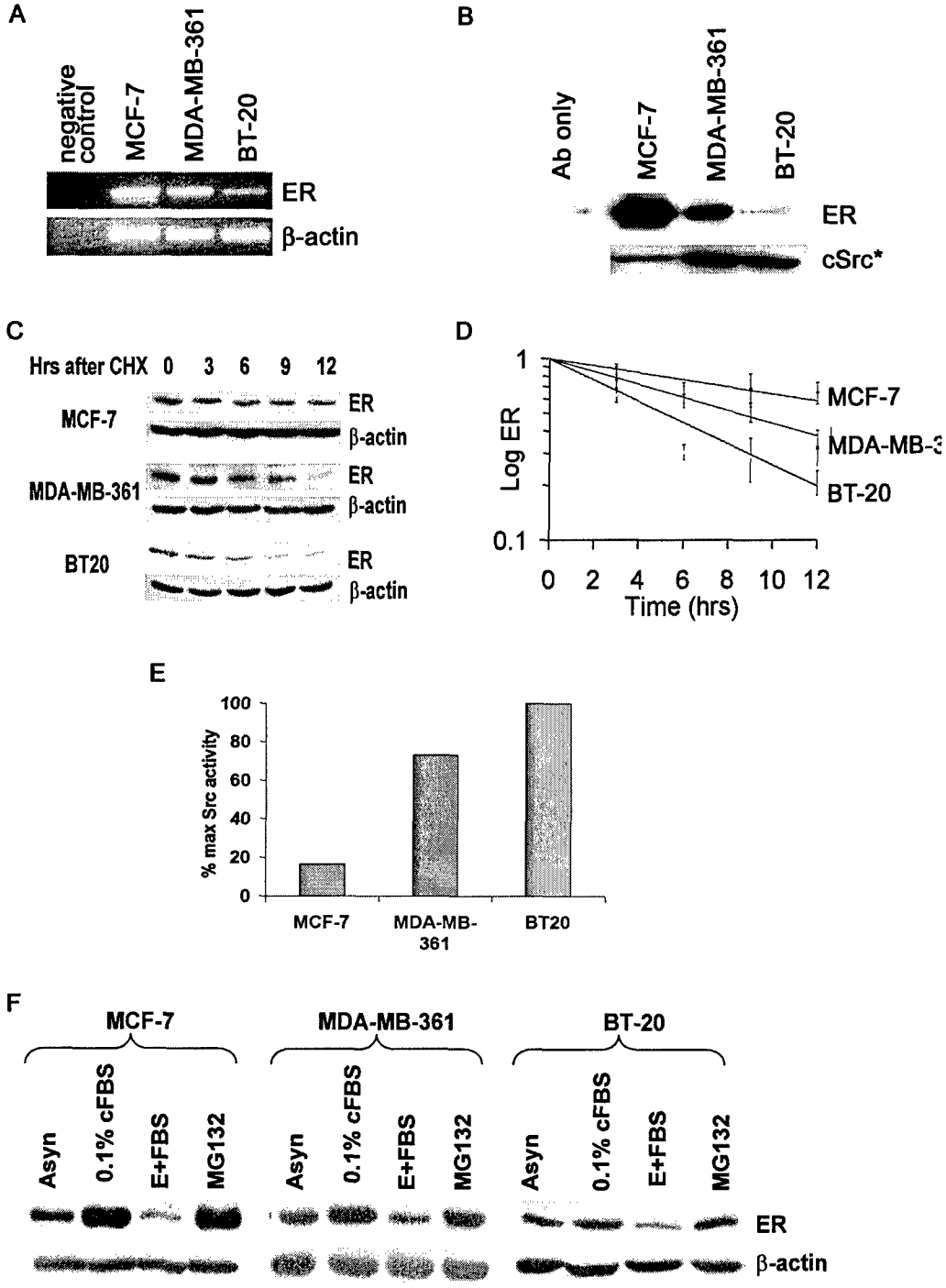

A
negative control
MCF-7
MDA-MB-361
BT-20
ER
β-actin
B
Ab only
MCF-7
MDA-MB-361
BT-20
ER
cSrc*
C
Hrs after CHX 0 3 6 9 12
MCF-7
ER
β-actin
MDA-MB-361
ER
β-actin
BT20
ER
β-actin
D
Log ER
1
0.1
0 2 4 6 8 10 12
Time (hrs)
MCF-7
MDA-MB-3
BT-20
E
% max Src activity
100
80
60
40
20
0
MCF-7
MDA-MB-361
BT20
F
MCF-7
MDA-MB-361
BT-20
Asyn
0.1% cFBS
E+FBS
MG132
ER
β-actin

Figure 6

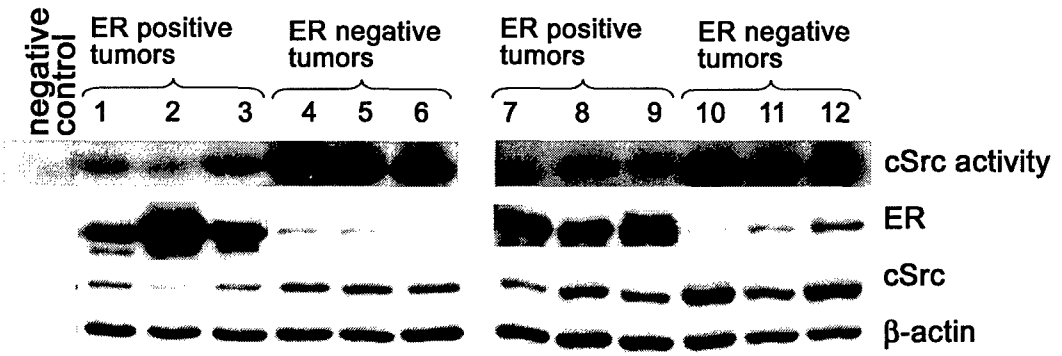

negative control
ER positive tumors
ER negative tumors
ER positive tumors
ER negative tumors
1 2 3 4 5 6 7 8 9 10 11 12
cSrc activity
ER
cSrc
β-actin

Figure 7